

Commentary

Linking xylem structure and function: the comparative method in from the cold

The field of functional xylem biology aims to understand how xylem structure gives rise to xylem function. This effort requires understanding processes at very tiny scales, from nanometric-level variation in the intricacies of pit membrane structure (Li *et al.*, 2016; Kaack *et al.*, 2021) to the intermolecular interactions involved in the formation of gas bubbles in xylem sap (Schenk *et al.*, 2015, 2017). It requires understanding how these very small-level events give rise to whole-plant properties, such as bulk water flow through the xylem or whole-plant transpiration and carbon (C) capture (Tyree & Ewers, 1991; Comstock & Sperry, 2000; Enquist *et al.*, 2007). It is necessary to understand how some individuals within populations in the distant past survived and reproduced better than their conspecifics all because of certain structure–function relations manifest at these different scales (Carlquist, 1975). This understanding extends to estimating forest productivity at a landscape level, modeling ‘average’ plant structure, or predicting which trees will die across a drought-stricken landscape (West *et al.*, 1999; Christoffersen *et al.*, 2016; Trugman *et al.*, 2021). Given the size of the task facing the field, the toolbox of xylem functional biology is a very large one indeed. The recent study published in this issue of *New Phytologist* by Savage *et al.* (2022; pp. 953–964) is a model of how this toolbox can, and must, seamlessly integrate data and theory from across levels, from the very small scale of processes within xylem conduits to the millions-plus years scale of evolutionary time. Before highlighting just some of the inferential richness in Savage *et al.* (2022), let me first explore why the integration of levels is such a challenge, and the empirical turn that this challenge demands.

Bottles of flies

Evolution by natural selection – the process leading to structure–function relationships – takes place within populations, making experimental studies (such as xylem physiology) and population biology the locus where structure–function mechanisms can be observed directly (Olson & Arroyo-Santos, 2015). Xylem physiologists use small parts of plants to observe as directly as possible the functional impact of variation in structure. Population biologists connect this knowledge of performance to heritable variation between members of a species and the fitness impacts of this heritable variation (Zobel & Buijtenen, 1989; Dalla-Salda *et al.*,

2011; Lauri *et al.*, 2011; Venturas *et al.*, 2014). The paradigm study system for population biology is *Drosophila* (Harshman & Hoffmann, 2000). With its short generation time and small size, thousands of flies can be subjected to detailed study of how variation in structure is related to performance and, ultimately, fitness. But as biologists, we want to understand the whole expanse of the living world, not just some flies in bottles. No matter how detailed, all studies of *Drosophila*, and of xylem physiology for that matter, suffer a fatal flaw: their tiny scale (Griffiths, 1996). Population biology and physiology experiments only examine a very small number of species, and even then only a small part of one or a few plants drawn from of a single population spanning the most minuscule ecological moment of that species. Nothing, so it seems, could be farther from the overall goal of biology to understand the living world’s protean variation. To see the very real relevance of observations at small scales, let us return now to Savage *et al.* (2022), who used the cold-deciduous forests of the north temperate zone as their study system.

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All vulnerabilities should be so lucky

The north temperate zone offers a useful model system for studying xylem structure–function relations because cold tolerance has received particular attention, and water conduction in the xylem is thought to play a key role in plant adaptation to cold (Zanne *et al.*, 2013). The way water freezes in the tiny compartments of plant conduits has been modeled admirably (Sevanto *et al.*, 2012). This understanding shows how, depending on temperature, conduit size, and solute concentration, gas comes out of solution as the sap freezes. These gas bubbles can have one of two fates. One is, as the xylem thaws, leaves are produced, and the conductive stream is once again under negative pressure, to expand until water movement in the entire conduit is impeded; that is, a bubble becomes an embolism. Another is to return to solution, no worse for its gas-phase winter wear, and no harm done either in the form of embolism. Moreover, colder temperatures and quicker freezing increase the likelihood of gas coming out of solution (Cavender-Bares *et al.*, 2005; Ball *et al.*, 2006; Sevanto *et al.*, 2012). This stark disjunction of either causing or

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not causing conduction-obstructing embolism has a dramatic manifestation in an apparent diameter threshold, which turns out to be $\approx 30 \mu\text{m}$, below which conduits tend to resist freezing-induced embolism and to succumb to it above (Davis *et al.*, 1999; Pittermann & Sperry, 2003, 2006; Cavender-Bares, 2005; Savage & Cavender-Bares, 2013).

Even an overview as perfunctory as the one I have just given is enough to show that freezing-induced embolism has a privileged place in functional xylem biology. This is because relatively few aspects of this level of importance in xylem structure–function relations are this well documented. Take, for example, the structural causes of drought-induced embolism. Society looks in vain to functional xylem biologists as millions of trees die or die back yearly due to climate-change induced drought; though the xylem is surely involved (Anderegg *et al.*, 2016; Adams *et al.*, 2017; Breshears *et al.*, 2018; Trugman *et al.*, 2021), as of yet the exact details of why variation in some structural features, such as conduit diameter, is associated with vulnerability to drought remain elusive. It seems clear that conduit diameter, pit membrane structure, wood density, conduit wall thickness, and pit membrane lipids, and others, are all potential contributors (Hacke *et al.*, 2001; Schenk *et al.*, 2017; Anfodillo & Olson, 2021; Kaack *et al.*, 2021). But this knowledge so far offers no tools for preventing forest dieback

because there is no mechanistic clarity comparable to that in the case of freezing-induced embolism.

But for all their precision, a model is just a model, and a physiology experiment is just an idyll examining a tiny fragment of a handful of individuals, not a species and certainly not anything at the scale of the processes shaping plants at large. What gives small-scale studies their relevance to understanding plant diversity at large is a simple assumption (Griffiths, 1996; Olson & Arroyo-Santos, 2015). As a community, xylem functional biologists assume that processes similar or identical to those being examined in physiology experiments are operative in other parts of an individual plant beyond the twig segments studied. They then assume that similar processes are operative in the other individuals of that species in the population. Then the big leap: they assume that similar processes have always been operative in the species, all the way back to its origin, and across other species as well. By doing so, observations of structure–function relations taken at a single moment from a tiny segment of a current population can be used to construct explanations regarding the action of natural selection in shaping structure–function relations observed across species today, exactly the goal of functional xylem biology. So, the relevance of these small-scale studies rests on a single key assumption, an assumption that cannot be tested within the confines of small-scale studies.



Fig. 1 Convergence as evidence of adaptation. Globular succulents are a celebrated example of convergent evolution. Globular succulents are close-to-spherical dryland plants found in several plant families and include the following examples: (a) Cactaceae, as in *Mammillaria polythele* in Hidalgo, Mexico; (b) Apocynaceae, as in *Pseudolithos migiurtinus* of Puntland (greater Somalia); (c) Euphorbiaceae, as in *Euphorbia meloformis* of South Africa's Eastern Cape, which experiences summer rain; or (d) Aizoaceae, as in *Conophytum* sp. of western South African quartz fields, where it experiences winter rain. Globular succulents are a celebrated example of convergence not because they conspicuously dominate the skyline or because are thought to provide major ecosystem services; most do not even cast any shade. Instead, they are celebrated because their form, which maximizes volume while minimizing surface area, is at once so unusual, so similar across families, and at the same time so obviously derived independently from ancestors that were not at all globular. Such very clear instances of convergence provide case studies that guide scientists through the more subtle processes molding plant life at large, most of which is more conventionally shaped than the plants shown here.

The deft empirical turn: the comparative method is the mother of all functional inferences

Assumptions need testing, and the assumption that underwrites small-scale studies such as physiology experiments and population biology is tested by an approach known as the comparative method (Olson, 2021). The comparative method for studying adaptation uses convergence as evidence consistent with the action of selection. Convergence is the observation that, in similar conditions of selection, similar organismal responses have evolved from ancestors with different trait values. There is good theoretical knowledge showing that spherical shapes minimize evaporative water loss, because a sphere has the minimal surface area for a given volume. Therefore, in conditions of low water availability, we would expect that within at least some species the individuals that are more sphere-like would survive and reproduce better than those with more surface area. This small-level information is consistent with the observation that in drylands there are indeed globular plants (Fig. 1). So, globular succulents like cacti, euphorbias, and stone plants are such a celebrated example of convergence because they so clearly came from ancestors that were not at all globular. In this way, comparative studies test the assumptions that give small-scale studies their relevance.

Savage *et al.* (2022) use the epistemological interdependence between small-scale and comparative studies to full advantage in connecting xylem structure to leaf phenology. Given detailed studies of few species plus models of freezing in conduits, Savage *et al.* (2022) knew that wider conduits are more vulnerable to freezing-induced embolism. From this they constructed the expectation that species with wider conduits should leaf out later in the spring, as the danger of frost lowers. Moreover, if 30 μm is a rough benchmark conduit diameter above which recovery from embolism is less likely, then species with twig conduits well above this threshold should leaf out the latest. So, Savage *et al.* (2022) clearly needed a wide range of twig conduit diameters to test this expectation.

Savage *et al.* (2022) focused on deciduous forests of the north temperate zone; with their low diversity from almost every metric imaginable, these forests would not seem to offer the most promising substrate for finding broad variation, but Savage *et al.* (2022) cleverly leveraged an ace that north temperate forests have up their sleeves. The forests of the north temperate zone are depauperate in practically every measure of biological diversity – brute species number per hectare, phylogenetic diversity (say at the ordinal level), diversity in plant life forms, range of wood density, even variation in leaf phenology (Edwards *et al.*, 2017) all brush up against global nadirs for angiosperm-dominated forests. Because virtually all biological traits are involved in multifarious associations, biological studies require variation in their independent variables, as much of it as possible, to detect associations with the variables of interest (Fig. 2). Given the need for maximal diversity on the x -axis, north temperate forests – remember, no baobabs, no ebonies or ironwoods, no balsa or papaya trees, practically no lianas, no trees with successive cambia, not even a decent attempt at a buttress (the list goes on) – might not seem like the most promising place to look. But Savage *et al.* (2022) remind us that north

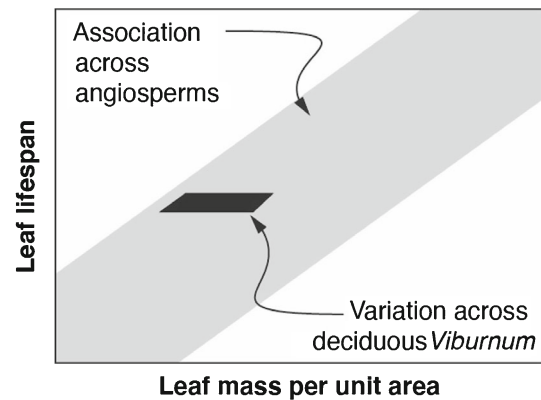


Fig. 2 The need for sampling wide ranges in the x variable in biological associations. Natural selection eliminates leaves with lifespans that are so short that they do not recuperate the costs of their construction (Kikuzawa & Lechowicz, 2011), hence the empty space at lower right in the leaf economics spectrum (high leaf mass per unit area (LMA) and short lifespan) (Onoda *et al.*, 2017). Maintenance costs become ever more costly with age even in highly productive low LMA leaves, hence the empty space at upper left (Kikuzawa & Lechowicz, 2006). In this way, natural selection maximizing leaf lifetime carbon gain leads to a marked association in leaf lifespan (LL) and LMA across species (Wright *et al.*, 2004). Though selection limits species to a relatively restricted region of the total space of possible trait combinations (gray shading), there is ample evolutionary leeway for variation in one trait given a single value in the other. Edwards *et al.* (2014) document a case in which diversification in a single genus occupies a very narrow range of LL (black shading). Like the species examined by Savage *et al.* (2022; pp. 953–964) in the recently published article in *New Phytologist*, these *Viburnum* species were all cold-adapted species of the north temperate zone. Edwards *et al.* (2014) found that different LMA values can evolve in the context of the same LL given variation in branching architecture in habitats subject to differing degrees of shade and cold. Like LL, which is predicted not just by LMA but also by habitat, architecture, and other traits as well, all biological traits are affected by multiple variables. Statistically, this means that there is variation about the axes in any relationship, hence the need to include as wide a range as possible in the x variable to detect the postulated association. Figure after Edwards *et al.* (2014).

temperate forests have a single claim to secondary xylem fame that saves them from being entirely subsumed within the great morphospace spanned by the rest of the world's flora: ring porosity.

North temperate redemption

For all their phenological uniformity (Edwards *et al.*, 2017) and low species diversity (Gillman *et al.*, 2015), some deciduous angiosperms of the north temperate zone are ring porous, the single saving grace that the north temperate zone can boast in response to the much higher variety in xylem structure in more varied latitudes. Virtually all growth ring types found in the north temperate zone can be found, plus so many others, in the tropics and the south temperate zone (Silva *et al.*, 2019). The exception is ring porosity (Fig. 3). Ring porosity is globally very exotic, being essentially restricted to the north temperate zone. It consists of a singular pattern, in which the first-formed earlywood vessels are extremely wide, quickly grading into narrow latewood vessels. It contrasts with the most common pattern globally, which is diffuse porosity (Wheeler *et al.*, 2007), in which vessel diameter varies scarcely to not at all across radial growth increments, or semi-ring porosity, in

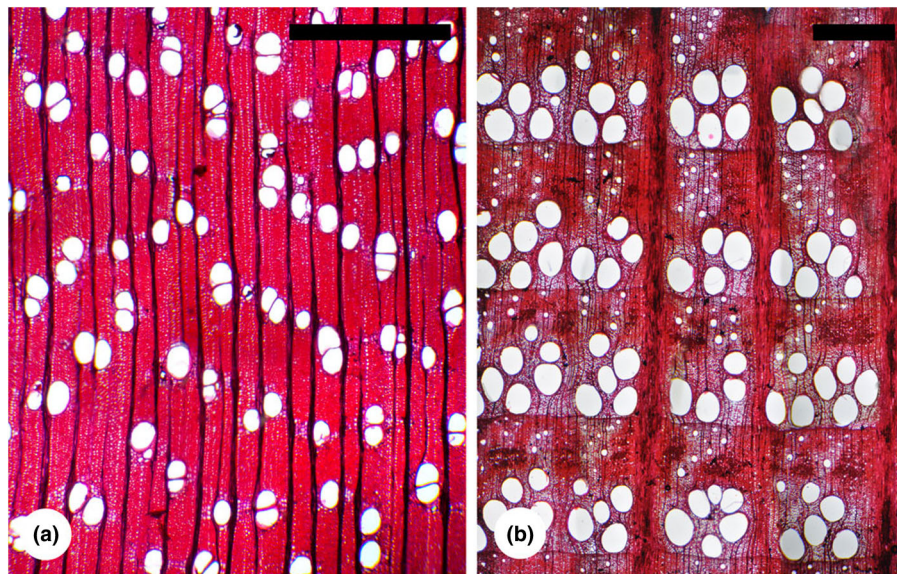


Fig. 3 Ring porosity: a curiosity of the north temperate zone. ‘Porosity’ refers to the ways that vessels (‘pores’ to foresters) vary throughout the year. (a) Most plants in the world are ‘diffuse porous’, having vessels that vary relatively little in diameter and density across the year, as in this *Ulmus mexicana* (Olson 4076). Across the very large diffuse-porous category, which goes from the tropics to the temperate zone, there is myriad variation in anatomy, presumably all of which is in some way coupled with some aspect of phenology. Many of these associations are likely extremely subtle, and thus difficult to detect; hence the wisdom of focusing on extreme variation. (b) Such extreme variation can be found in ring-porous species, which contrast conspicuously with diffuse-porous species in having very wide first-formed earlywood vessels that quickly give way to narrow mid to late-wood vessels, as in *Quercus rubra* (Olson 5075). Ring-porous species are not common globally and almost entirely restricted to relatively few species of the north temperate zone. Including them in a study that also includes diffuse porosity throws into relief the ways that marked variation in xylem structure is associated with variation in phenology, paving the way for studies that span the global range of xylem variation. Bars, 1 mm.

which vessel diameter or abundance (or both) decrease from earlywood to latewood, but without the massive earlywood vessels and abrupt transition to narrower vessels characteristic of ring porosity. That remarkable resource, the InsideWood database (insidewood.lib.ncsu.edu), shows that in all of Australia there are essentially no ring-porous species. In the vastness and superlative floristic richness that is tropical South America, the database reveals not a single paradigm example of ring porosity; the same for tropical Southeast Asia, the other global apogee of plant floristic diversity. In fact, not only the equatorial tropics but also the entire Southern Hemisphere are all essentially innocent of ring porosity. So, ring porosity is definitely globally out of the ordinary, much rarer than ‘exotic’ xylem morphologies such as water-storing ‘bottle trees’ or woody plants with successive cambia.

The span of porosity from diffuse to ring types provided exactly the range of vessel diameters that Savage *et al.* (2022) needed. Quantifying leaf-out time across 87 north temperate species, this range revealed exactly the predicted pattern: species with vessels that are wider and presumably more vulnerable to freezing-induced embolism leaf out later in the season. As they predicted, species with vessels wider than the 30 μm threshold only leafed out after the risk of freezing was well past. If, within a population of a deciduous north temperate species, there is variation in leaf-out time for a given vessel diameter, then individuals that leaf out so early that their vessels are exposed to embolism will suffer the metabolic cost that recovery implies. Individuals that leaf out very late will be spared freezing-induced embolism but lose valuable photosynthetic time. The variants that fix the most C, and thus become

disproportionately represented in subsequent generations, should therefore be those that leaf out just early enough so that most risk of freezing is dodged given vessel diameter. In this way, with their clever use of the ring-to-diffuse porosity span, Savage *et al.* (2022) connect the microscale processes operating within conduits to outcomes at the interspecific scale, precisely the interdependence necessary for robust explanations of xylem function. Their inferences do not stop there.

Inferential developments

Savage *et al.*'s (2022) use of the comparative method even leads to testable predictions regarding ontogeny. They cite studies showing that diffuse-porous species (Fig. 3) supply leafing-out with existing vessels, producing new vessels synchronously with leaves, and that ring-porous species first produce earlywood conduits and then leaf out. They note that greater sensitivity to auxin could cause this difference, with diffuse-porous species requiring greater levels of auxin to produce new vessels, hence their producing new vessels only after leaf maturation. Illustrating the deductive power of the comparative method, this assertion implies two alternative and entirely testable predictions. The first implies that, within a diffuse-porous species, earlier or later leaf-out times for a given vessel diameter must be developmentally impossible, even under hormonal or other manipulation. Because variation is necessary for selection to occur, if variation is impossible then the observed trait value cannot be explained by appeal to selection (Alberch, 1989; Brakefield, 2006; Frankino

et al., 2010; Badyaev, 2011). If, on the other hand, the correlation between leaf-out time and vessel diameter is due to selection, it implies that leaf-out time can vary relative to vessel development but that selection eliminates variants that leaf-out 'too early' or 'too late'. The latter seems much more likely, if for no other reason than it is impossible to explain why leaf-out time correlates so strongly with vessel diameter across species if leaf-out time and vessel diameter are linked developmentally such that variation is impossible. Instead, if they are free to vary independently across the individuals of a population, then selection can tune the leaf-out time–vessel diameter fit. Whatever the merits of this argument, a fully supported explanation requires testing the expectation that leaf-out time and xylem development can vary mutually. Savage *et al.* (2022) give us exactly the necessary direction for performing these tests, further underscoring the power of the comparative method.


Conclusion: togetherness in the cold

That the comparative method can generate both predictions regarding the outcomes of selection between varying individuals within populations and even expectations regarding the individual-level potentialities of ontogeny is illustration enough of its inferential reach. But most importantly of all, it is the only source of direct evidence that shows that the processes operative at the population level can indeed plausibly be regarded as shaping the whole diversity of life. As such, xylem physiologists, population biologists, and optimality modelers must necessarily ally with the comparative method. Using the harsh conditions of cold on the deciduous trees of the north temperate zone, Savage *et al.* (2022) provide a roadmap of just how this integration of layers of direct evidence must be forged.

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